Stabilization of GFP Expression in a Transient Expression System using Viral Suppressors of Silencing

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ABSTRACT

Using transient expression systems, introduction of the *green fluorescent protein* (*gfp*) gene results in peak GFP expression 24 hrs post-bombardment (hpb), followed by a rapid decline within 72 hpb. The decline in GFP expression during transient expression probably results from the silencing of the *gfp* gene. Gene silencing or RNA silencing were developed by plants as a mechanism to combat viruses. As a counter measure, viruses encode certain proteins that suppress the silencing mechanism of plants. We evaluated six viral "suppressors of silencing" for their abilities to stabilize GFP expression using a transient expression system. Suppressor constructs were generated to allow introduction of the suppressor as a fusion with the *gfp* gene or on a separate plasmid from the *gfp* gene. Various suppressor constructs were introduced into lima bean (*Phaseolus lunatus* L.) cotyledons from germinating seedlings, using particle bombardment. Post-introduction, GFP expression was tracked over time using an automated image collection and analysis system. The silencing suppressors HC-Pro and p19 stabilized GFP expression when introduced as 3´ *gfp* translational fusions, while p21 and γb stabilized GFP expression when co-introduced with the *gfp* gene on separate plasmids. The last two suppressors, AL2 and replicase, did not stabilize GFP expression in lima bean system. Introduction of the 3´ *gfp* fusion of p19 (35S-*gfp*::p19-nos) into soybean (*Glycine max* (L.) Merrill) resulted in recovery of plants with an abnormal downward-leaf curling phenotype, suggesting that p19 not only affects the expression of the gene it is fused with, but also expression of other genes *in-trans*.

INTRODUCTION

Gene transfer can be used to study gene expression or produce transgenic organisms with desired novel characteristics. However, transgene introduction often leads to variable levels of expression (1). RNA silencing is one of the many reasons that can lead to highly variable transgene expression (3). Recent studies have shown that plants employ RNA silencing as an anti-viral defense mechanism (6). To counter the plant's defense system, plant virus genomes encode proteins, called suppressors of silencing that suppress the silencing mechanism of plants.

Different silencing suppressors can affect different points in the silencing pathway (2). Suppressors like HC-Pro, p19 and p21 can inhibit the production of short-interfering RNAs (siRNAs) or their incorporation into the RNA induced silencing complex (RISC) and thus prevent post-transcriptional gene silencing (PTGS). AL2 inhibits the methylation of the virus genome in the plant cell nucleus, preventing transcriptional gene silencing (TGS).

MATERIALS and METHODS

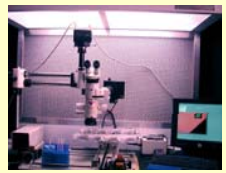
Transient Expression: Six viral suppressors of silencing (Table 1) were evaluated for their abilities to stabilize GFP expression in a transient system. The viral suppressors were introduced into lima bean cotyledons as 3´ *gfp* fusions (Fig. 1a) or co-introduced with the *gfp* gene on separate plasmids (Fig. 1b) via particle bombardment. GFP expression in the cotyledons was captured and quantified for over 100 hours using a robotics system and image analysis software.

Fig 1. DNA constructions for bombardment experiments. (a) represents a fusion construction (35S-*gfp*::suppressor-nos) and (b) represents DNA constructions used for co-introduction experiments (35S-*gfp*-nos + 35S-Suppressor-nos)

Table 1. Various suppressors of silencing and their source

Suppressors of silencing	Virus
HC-Pro	Tobacco etch virus
p19	Tomato bushy stunt virus
AL2	Tomato golden mosaic virus
p21	Beet yellows virus
γb	Barley stripe mosaic virus
Replicase	Tobacco mosaic virus

Lima bean seeds harvested, sterilized and germinated → Cotyledons excised and placed on media without hormones → DNA introduction into cotyledons via particle bombardment (4) → Cotyledons placed back on media and Petri dishes placed on robot for tracking GFP expression



Robotics system consisting of a two dimensional robotics platform, a fluorescence dissecting microscope and digital camera, all under computer control and located in a laminar air flow hood (5)

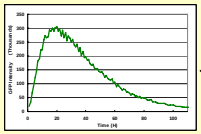


Image analysis using Image J software ← Automated RGB (red, green, blue) image collection of GFP expression by digital camera.

Stable Expression: The 35S-*gfp*::p19-nos and 35S-Hytru-nos were co-introduced into soybean embryogenic tissue to produce stable transformation events. Post-bombardment, transgenic events were selected for by placing the tissue in hygromycin-containing media and plants were regenerated. T1 generation plants were grown to study the segregation of GFP expression with the phenotype.

RESULTS

Transient Expression

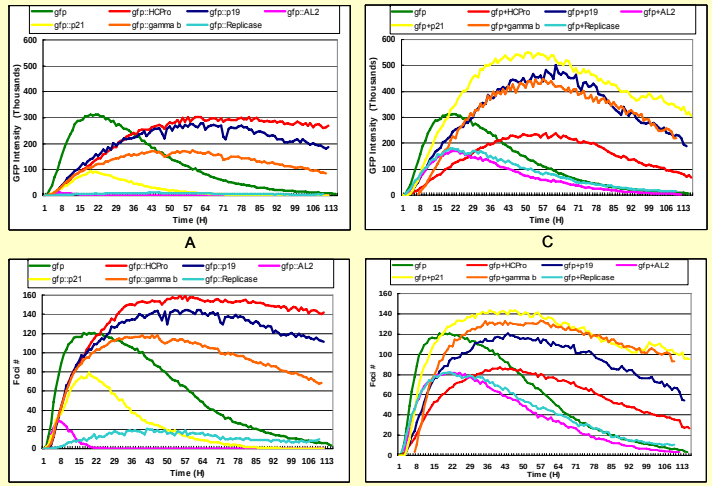


Fig 2. Quantification of GFP expression in the presence of various silencing suppressors, introduced either as 3´ *gfp* fusions (A, B) or co-introduced with the *gfp* gene on separate plasmids (C, D) into lima bean cotyledons

Stable Expression

Introduction of 35S-*gfp*::p19-nos in soybean embryogenic tissue resulted in downward leaf-curling phenotype in plants from one transformation event. In the T1 generation, the phenotype did not segregate with GFP expression.

Fig 3. (a) Comparison of plants with normal and abnormal phenotypes in the T1 generation. (b) Closer view of altered leaf morphology in T1 generation

CONCLUSIONS and DISCUSSION

- HC-Pro and p19 stabilized GFP expression as 3´ *gfp* fusions for over 100 hrs post-bombardment..
- p21 and γb prolonged GFP expression when co-introduced with the *gfp* gene on separate plasmids.
- Replicase and AL2 did not stabilize GFP expression in the lima bean model system.
- Introduction of 35S-*gfp*::p19-nos in soybean embryogenic tissue resulted in recovery of plants with a downward leaf-curling phenotype, suggesting that p19 may not only affect the expression of the gene it is fused with, but also other developmentally-regulated genes. Similar phenotype was also reported in *Nicotiana benthamiana* due to p19 (7).
- Variation in the phenotypes indicates a dosage-effect of p19.

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